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Chapter 1

Non-Viral Delivery Systems in Gene Therapy

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Additional information is available at the end of the chapter

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1. Introduction

Recent advances in molecular biology combined with the culmination of the Human Genome Project [1] have provided a genetic understanding of cellular processes and disease pathogenesis; numerous genes involved in disease and cellular processes have been identified as targets for therapeutic approaches. In addition, the development of high-throughput screening techniques (e.g., cDNA microarrays, differential display and database meaning) may drastically increase the rate at which these targets are identified [2,3]. Over the past years there has been a remarkable expansion of both the number of human genes directly associated with disease states and the number of vector systems available to express those genes for therapeutic purposes. However, the development of novel therapeutic strategies using these targets is dependent on the ability to manipulate the expression of these target genes in the desired cell population. In this chapter we explain the concept and aim of gene therapy, the different gene delivery systems and therapeutic strategies, how genes are delivered and how they reach the target.

2. Aim and concept of gene therapy with non-viral vectors

A gene therapy medicinal product is a biological product which has the following characteristics: (a) it contains an active substance which contains or consists of a recombinant nucleic acid used in administered to human beings with a view to regulating, repairing, replacing, adding or deleting a genetic sequence; (b) its therapeutic, prophylactic or diagnostic effect relates directly to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence [4].

The most important, and most difficult, challenge in gene therapy is the issue of delivery. The tools used to achieve gene modification are called gene therapy vectors and they are the "key" for an efficient and safe strategy. Therefore, there is a need for a delivery system, which must first overcome the extracellular barriers (such as avoiding particle clearance mechanisms, targeting specific cells or tissues and protecting the nucleic acid from degradation) and, subsequently, the cellular barriers (cellular uptake, endosomal escape, nuclear entry and nucleic release) [5]. An ideal gene delivery vector should be effective, specific, long lasting and safe.

Gene therapy has long been regarded a promising treatment for many diseases, including inherited through a genetic disorder (such as hemophilia, human severe combined immunodeficiency, cystic fibrosis, etc) or acquired (such as AIDS or cancer). Figures 1 and 2 show the indications addressed and the gene types transferred in gene therapy clinical trials, respectively [6].

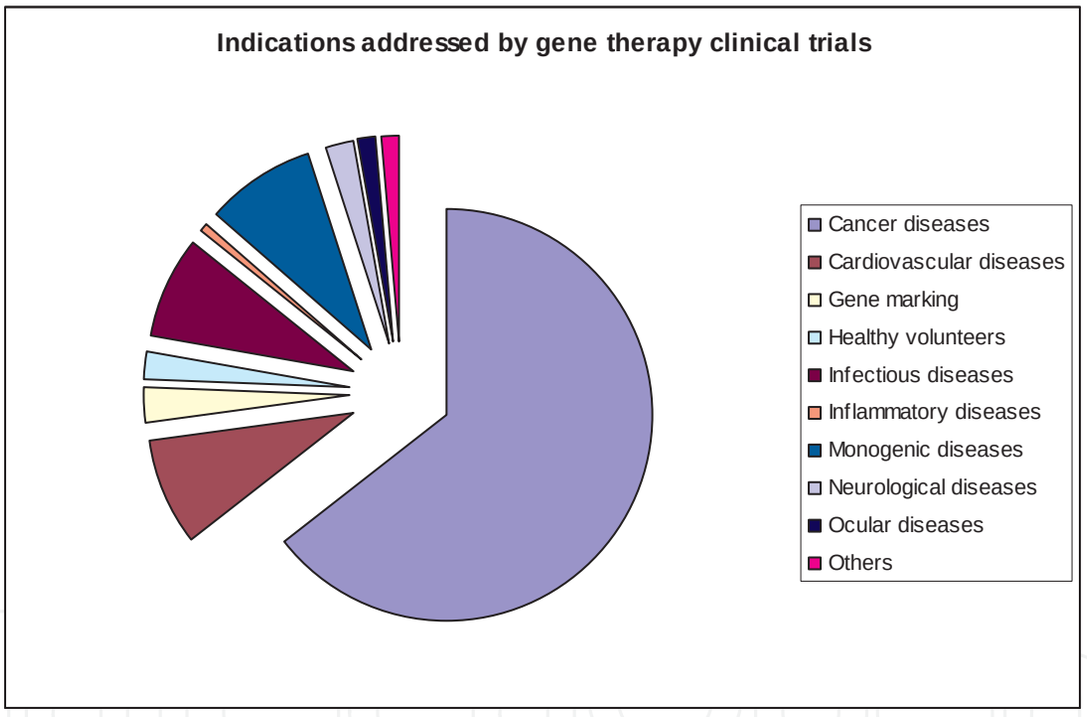

Figure 1. Indications addressed by gene therapy clinical trials (adapted from http://www.wiley.co.k/genmed/clinical).

Gene delivery systems include viral vectors and non-viral vectors. Viral vectors are the most effective, but their application is limited by their immunogenicity, oncogenicity and the small size of the DNA they can transport. Non-viral vectors are safer, of low cost, more reproducible and do not present DNA size limit. The main limitation of non-viral systems is their low transfection efficiency, although it has been improved by different strategies and the efforts are still ongoing [6]; actually, advances of non-viral delivery have lead to an increased number of products entering into clinical trials. However, viral vector has dominated the clinical trials in gene therapy for its relatively high delivery efficiency. Figure 3 shows the proportion of vector systems currently in human trials [7].

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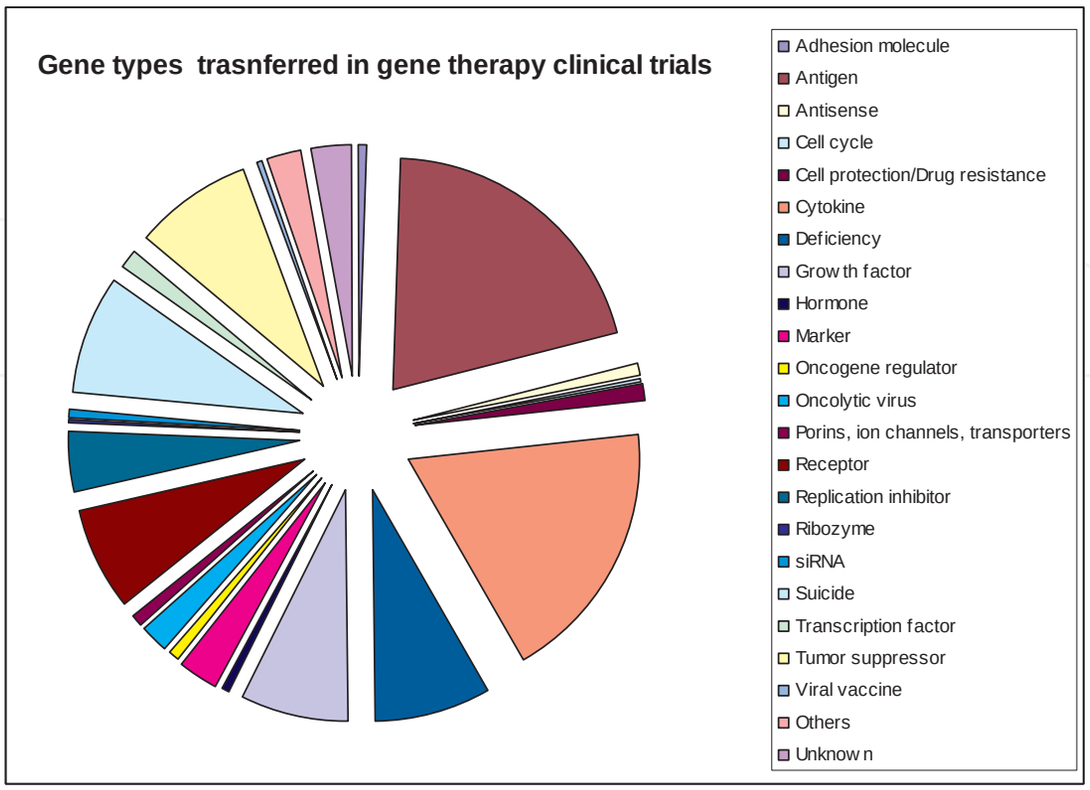

Figure 2. Gene types transferred in gene therapy clinical trials (adapted from http://www.wiley.co.k/genmed/clinical).

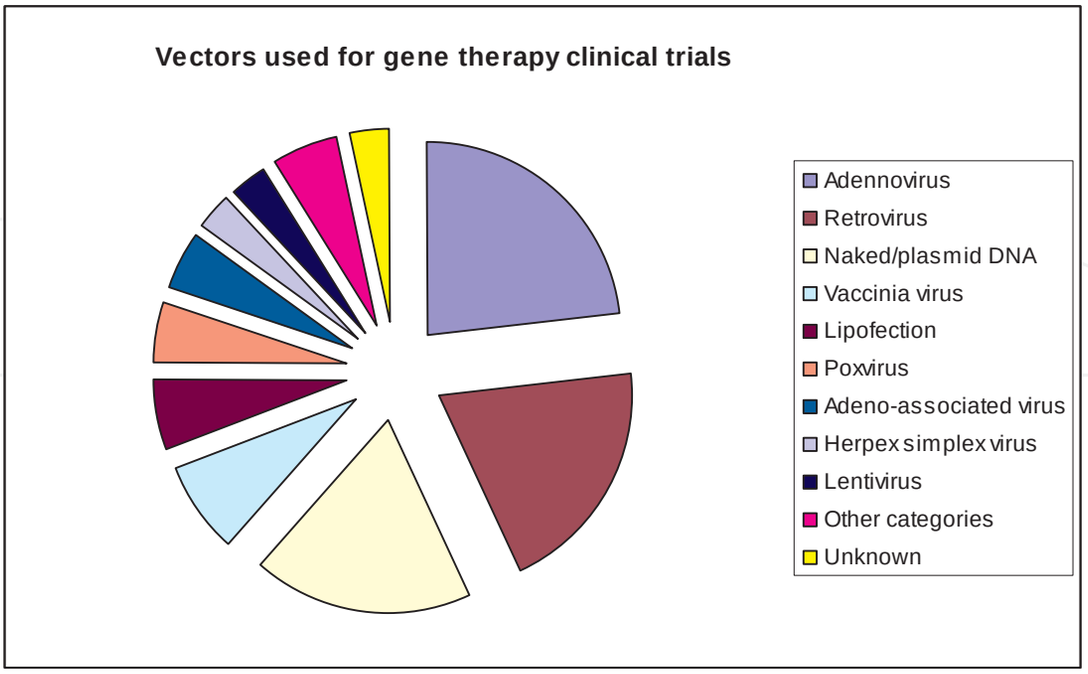

Figure 3. Vector systems used in gene therapy clinical trials (adapted from http://www.wiley.co.k/genmed/clinical).

3. Non-viral methods for transfection

Currently, three categories of non-viral systems are available:

- Inorganic particles
- Synthetic or natural biodegradable particles
- Physical methods

Table 1 summarizes the most utilized non-viral vectors.

Category	System for gene delivery
Inorganic particles	Calcium phosphate
	Silica
	Gold
	Magnetic
Synthetic or natural biodegradable particles	1. Polymeric-based non-viral vectors:
	Poly(lactic-co-glycolic acid) (PLGA)
	Poly lactic acid (PLA)
	Poly(ethylene imine) (PEI)
	Chitosan
	Dendrimers
	Polymethacrylates
	2. Cationic lipid-based non-viral vectors:
	Cationic liposomes
	Cationic emulsions
	Solid lipid nanoparticles
	3. Peptide-based non-viral vectors:
	Poly-L-lysine
	Other peptides to functionalize other delivery systems: SAP, protamine
Physical methods	Needle injection
	Balistic DNA injection
	Electroporation
	Sonoporation
	Photoporation
	Magnetofection
	Hydroporation

Table 1. Delivery systems for gene therapy.

3.1. Inorganic particles

Inorganic nanoparticles are nanostructures varying in size, shape and porosity, which can be engineered to evade the reticuloendothelial system or to protect an entrapped molecular payload from degradation or denaturation [8]. Calcium phosphate, silica, gold, and several magnetic compounds are the most studied [9-11]. Silica-coated nanoparticles are biocompatible structures that have been used for various biological applications including gene therapy due to its biocompatibility [8]. Mesoporous silica nanoparticles have shown gene transfection efficiency "in vitro" in glial cells [12]. Magnetic inorganic nanoparticles (such as Fe_3O_4, MnO_2) have been applied for cancer-targeted delivery of nucleic acids and simultaneous diagnosis via magnetic resonance imaging [13,14]. Silica nanotubes have been also studied as an efficient gene delivery and imaging agent [13].

Inorganic particles can be easily prepared and surface-functionalized. They exhibit good storage stability and are not subject to microbial attack [13]. Bhattarai et al. [15] modified mesoporous silica nanoparticles with poly(ethylene glycol) and methacrylate derivatives and used them to deliver DNA or small interfering RNA (siRNA) "in vitro".

Gold nanoparticles have been lately investigated for gene therapy. They can be easily prepared, display low toxicity and the surface can be modified using various chemical techniques [16]. For instance, gold nanorods have been proposed to deliver nucleic acids to tumors [13]. They have strong absorption bands in the near-infrared region, and the absorbed light energy is then converted into heat by gold nanorods (photohermal effect). The near-infrared light can penetrate deeply into tissues; therefore, the surface of the gold could be modified with double-stranded DNA for controlled release [17]. After irradiation with near-infrared light, single stranded DNA is released due to thermal denaturation induced by the photothermal effect.

3.2. Synthetic or natural biodegradable particles

Synthetic or natural biocompatible particles may be composed by cationic polymers, cationic lipids or cationic peptides, and also the combination of these components [18-21]. The potential advantages of biodegradable carriers are their reduced toxicity (degradation leads to non-toxic products) and avoidance of accumulation of the polymer in the cells.

3.2.1. Polymer-based non-viral vectors

Cationic polymers condense DNA into small particles (polyplexes) and prevent DNA from degradation. Polymeric nanoparticles are the most commonly used type of nano-scale delivery systems. They are mostly spherical particles, in the size range of 1-1000 nm, carrying the nucleic acids of interest. DNA can be entrapped into the polymeric matrix or can be adsorbed or conjugated on the surface of the nanoparticles. Moreover, the degradation of the polymer can be used as a tool to release the plasmid DNA into the cytosol [22]. Table 1 shows several commonly used polymers used for gene delivery [16].

3.2.1.1. Poly(lactic-co-glycolic acid) (PLGA) and poly lactic acid (PLA)

Biodegradable polyesters, PLGA and PLA, are the most commonly used polymers for delivering drugs and biomolecules, including nucleic acids. They consist of units of lactic acid and glycolic acid connected through ester linkage. These biodegradable polymers undergo bulk hydrolysis thereby providing sustained delivery of the therapeutic agent. The degradation products, lactic acid and glycolic acid, are removed from the body through citric acid cycle. The release of therapeutic agent from these polymers occurs by diffusion and polymer degradation [16].

PLGA has a demonstrated FDA approved track record as a vehicle for drug and protein delivery [23,24]. Biodegradable PLA and PLGA particles are biocompatible and have the capacity to protect pDNA from nuclease degradation and increase pDNA stability [25,26].

PLGA particles typically less than 10 μm in size are efficiently phagocytosed by professional antigen presenting cells; therefore, they have significant potential for immunization applications [27,28]. For example, intramuscular immunization of p55 Gag plasmid adsorbed on PLGA/cetyl trimethyl ammonium bromide (CTAB) particles induced potent antibody and cytotoxic T lymphocyte responses. These particles showed a 250-fold increase in antibody response at higher DNA doses and more rapid and complete seroconversion, at the lower doses, compared to other adjuvants, including cationic liposomes [29].

The encapsulation efficiency of DNA in PLGA nanoparticles is not very high, and it depends on the molecular weight of the PLGA and on the hydrophobicity of the polymer, being the hydrophilic polymers those that provide higher loading efficiency [30]. To enhance the DNA loading, several strategies have been proposed. Kusonowiriyawong et al. [31] prepared cationic PLGA microparticles by dissolving cationic surfactants (like water insoluble stearylamine) in the organic solvent in which the PLGA was dissolved to prepare the microparticles. Another strategy was to reduce the negative charge of plasmid DNA by condensing it with poly(aminoacids) (like poly-L-lysine) before encapsulation in PLGA microparticles [32,33].

Normally, after an initial burst release, plasmid DNA release from PLGA particles occurs slowly during several days/weeks [22]. The degradation of the PLGA nanoparticles, through a bulk homogeneous hydrolytic process, determines the release of plasmid DNA. Consequently, it can be expected that the use of more hydrophilic PLGA not only improves the encapsulation efficiency of DNA, but also results in a faster release of plasmid DNA. Delivery of the plasmid DNA depends on the copolymer composition of the PLGA (lactic acid versus glycolic acid), molecular weight, particle size and morphology [22]. DNA release kinetics depends also on the plasmid incorporation technique; Pérea et al. [34] reported that nanoparticles prepared by the water-oil emulsion/diffusion technique released their content rapidly, whereas those obtained by the water-oil-emulsion method showed an initial burst followed by a slow release during at least 28 days.

PLGA and PLA based nanoparticles have also been used for “in vitro” RNAi delivery [35]. For instance, Hong et al. [36] have shown the effects of glucocorticoid receptor siRNA deliv-

ered using PLGA microparticles, on proliferation and differentiation capabilities of human mesenchymal stromal cells.

3.2.1.2. Chitosan

Chitosan [b(1-4)2-amino-2-deoxy-D-glucose] is a biodegradable polysaccharide copolymer of N-acetyl-D-glucosamine and D-glucosamine obtained by the alkaline deacetylation of chitin, which is a polysaccharide found in the exoskeleton of crustaceans of marine arthropods and insects [37]. Chitosans differ in the degree of N-acetylation (40 to 98%) and molecular weight (50 to 2000 kDa) [38]. As the only natural polysaccharide with a positive charge, chitosan has the following unique properties as carrier for gene therapy:

- it is potentially safe and non-toxic, both in experimental animals [39] and humans [40]
- it can be degraded into H_2O and CO_2 in the body, which ensures its biosafety
- it has biocompatibility to the human body and does not elicit stimulation of the mucosa and the derma
- its cationic polyelectrolyte nature provides a strong electrostatic interaction with negatively charged DNA [41], and protects the DNA from nuclease degradation [42]
- the mucoadhesive property of chitosan potentially leads to a sustained interaction between the macromolecule being "delivered" and the membrane epithelia, promoting more efficient uptake [43]
- it has the ability to open intercellular tight junctions, facilitating its transport into the cells [44]

Currently, there is a commercial transfection reagent based on chitosan (Novafect, NovaMatrix, FMC, US), and many other prototypes are under development. Most of the chitosan-based nanocarriers for gene delivery have been based on direct complexation of chitosan and the nucleic acid [45], whereas in some instances additional polyelectrolytes, polymers and lipids have been used in order to form composite nanoparticles [46-49] or chitosan-coated hydrophobic nanocarriers.

Many studies using cell cultures have shown that pDNA-loaded chitosan nanocarriers are able to achieve high transfection levels in most cell lines [50]. Chitosan nanocarriers loaded with siRNA have provided gene suppression values similar to the commercial reagent lipofectamine [51,52,18,53].

Chitosan of low molecular weight is more efficient for transfection than chitosan with high molecular weight. This enhancement in transfection efficacy observed with low molecular weight chitosan can be attributed to the easier release of pDNA from the nanocarrier upon cell internalization. Moreover, the presence of free low molecular chitosan has been deemed to be very important for the endosomal escape of the nanocarriers [50]. Concerning deacetylation degree, its influence on transfection is not still clear. "In vitro" studies have shown that the best transfection is achieved with highly deacetylated chitosan [54,55]. However, "in

vivo", higher transfection was achieved after intramuscular administration of chitosan complexes with a low deacetylation degree [55].

3.2.1.3. Poly(ethylene imine) (PEI)

PEI is one of the most potent polymers for gene delivery. PEI is produced by the polymerization of aziridine and has been used to deliver genetic material into various cell types both "in vitro" and "in vivo" [56,57]. There are two forms of this polymer: the linear form and the branched form, being the branched structure more efficient in condensing nucleic acids than the linear PEI [58].

PEI has a high density of protonable amino groups, every third atom being amino nitrogen, which imparts a high buffering ability at practically any pH [16]. Hence, once inside the endosome, PEI disrupts the vacuole releasing the genetic material in the cytoplasm. This ability to escape from the endosome, as well as the ability to form stable complexes with nucleic acids, make this polymer very useful as a gene delivery vector [56].

Depending on the type of polymer (e.g. linear or branched PEI), as well as the molecular weight, the particle sizes of the polyplexes formed are more or less uniformly distributed [59]. Transfection efficiency of PEI has been found to be dependent on a multitude of factors such as molecular weight, degree of branching, N/P ratio, complex size, etc [60].

The use of PEI for gene delivery is limited due to the relatively low transfection efficiency, short duration of gene expression, and elevated toxicity [61,62]. Conjugation of poly(ethylene glycol) to PEI to form diblock or triblock copolymers has been used by some authors to reduce the toxicity of PEI [63,64,65]. Poly(ethylene glycol) also shields the positive charge of the polyplexes, thereby providing steric stability to the complex. Such stabilization prevents non-specific interaction with blood components during systemic delivery [66].

3.2.1.4. Dendrimes

Dendrimers are polymer-based molecules with a symmetrical structure in precise size and shapes, as well as terminal group functionality [8]. Dendrimers contain three regions: i) a central core (a single atom or a group of atoms having two or more identical chemical funcionalities); ii) branches emanating from core, which are composed of repeating units with at least one branching junction, whose repetition is organized in a geometric progression that results in a series of radially concentric layers; and iii) terminal function groups. Dendrimers bind to genetic material when peripheral groups, that are positively-charged at physiological pH, interact with the negatively-charged phosphate groups of the nucleic acid [67,68]. Due to their nanometric size, dendrimers can interact effectively and specifically with cell components such as membranes, organelles, and proteins [69].

For instance, Qi et al. [70] showed the ability of generations 5 and 6 (G5 and G6) of poly(amidoamine) (PAMAM) dendrimers, conjugated with poly(ethylene glycol) to efficiently transfect both "in vitro" and "in vivo" after intramuscular administration to neonatal mice. PAMAM has also the ability to deliver siRNAs, especially "in vitro" in cell culture sys-

tems [71-73]. Recent studies showed that the dendrimer-mediated siRNA delivery and gene silencing depends on the stoichiometry, concentration of siRNA and the dendrimer generation [71]. In a recent study, a PAMAM dendrimer-delivered short hairpin RNA (shRNA) showed the ability to deplect a human telomerase reverse transcriptase, the catalytic subunit of telomerase complex, resulting in partial cellular apoptosis, and inhibition of tumor outgrowth in xenotransplanted mice [74].

The toxicity profile of dendrimers is good, although it depends on the number of terminal amino groups and positive charge density. Moreover, toxicity is concentration and generation dependent with higher generations being more toxic as the number of surface groups doubles with increasing generation number [75,76].

3.2.1.5. Polymethacrylates

Polymethacrylates are cationic vinyl-based polymers that possess the ability to condense polynucleotides into nanometer size particles. They efficiently condense DNA by forming inter-polyelectrolyte complexes. A range of polymethacrylates, differing in molecular weights and structures, have been evaluated for their potential as gene delivery vector, such us poly[2-dimethylamino) ethyl methacrylate] (DMAEMA) and its co-polymers [16]. The use of polymethactrtylates for DNA transfection is, however, limited due to their low ability to interact with membranes.

In order to optimise the use of these compounds for gene transfer, Christiaens et al. [77] combined polymethacrylates with penetratin, a 16-residue water-soluble peptide that internalises into cells through membrane translocation. Penetratin mainly enhanced the endolysosomal escape of the polymethacrylate–DNA complexes and increased their cellular uptake using COS-1 (kidney cells of the African green monkey). Nanoparticles with a methacrylate core and PEI shell prepared via graft copolymerization have also been employed lately for gene delivery [78,79]. This conjugation resulted in nanoparticles with a higher transfection efficiency and lower toxicity as compared with PEI.

3.2.2. Cationic-lipid based non-viral vectors

Cationic lipids have been among the more efficient synthetic gene delivery reagents “in vitro” since the landmark publications in the late 1980s [80]. Cationic lipids can condense nucleic acids into cationic particles when the components are mixed together. This cationic lipid/nucleic acid complex (lipoplex) can protect nucleic acids from enzymatic degradation and deliver the nucleic acids into cells by interacting with the negatively charged cell membrane [81]. Lipoplexes are not an ordered DNA phase surrounded by a lipid bilayer; rather, they are a partially condensed DNA complex with an ordered substructure and an irregular morphology [82,83]. Since the initial studies, hundreds of cationic lipids have been synthesized as candidates for non-viral gene delivery [84] and a few made it to clinical trials [85,86].

Cationic lipids can be used to form lipoplexes by directly mixing the positively charged lipids at the physiological pH with the negatively charged DNA. However, cationic lipids are

more frequently used to prepare lipoplex structures such as liposomes, nanoemulsions or solid lipid nanoparticles [81].

3.2.2.1. Cationic liposomes

Liposomes are spherical vesicles made of phospholipids used to deliver drugs or genes. They can range in size from 20 nm to a few microns. Cationic liposomes and DNA interact spontaneously to form complexes with 100% loading efficiency; in other words, all of the DNA molecules are complexed with the liposomes, if enough cationic liposomes are available. It is believed that the negative charges of the DNA interact with the positively charged groups of the liposomes [87]. The lipid to DNA ratio, and overall lipid concentration used in forming these complexes, are very important for efficient gene delivery and vary with applications [88].

Liposomes offer several advantages for gene delivery [87]:

- they are relatively cheap to produce and do not cause diseases
- protection of the DNA from degradation, mainly due to nucleases
- they can transport large pieces of DNA
- they can be targeted to specific cells or tissues

Successful delivery of DNA and RNA to a variety of cell types has been reported, including tumour, airway epithelial cells, endothelial cells, hepatocytes, muscle cells and others, by intratissue or intravenous injection into animals [89,90].

Several liposome-based vectors have been assayed in a number of clinical trials for cancer treatment. For instance, Allovectin-7® (Vical, San Diego, CA, USA), a plasmid DNA carrying HLAB and ß2-microglobulin genes complexed with DMRIE/DOPE liposomes have been assessed for safety and efficacy in phase I and II clinical trials [91,92].

3.2.2.2. Lipid nanoemulsions

An emulsion is a dispersion of one immiscible liquid in another stabilized by a third component, the emulsifying agent [93]. The nanoemulsion consists of oil, water and surfactants, and presents a droplet size distribution of around 200 nm. Lipid-based carrier systems represent drug vehicles composed of physiological lipids, such as cholesterol, cholesterol esters, phospholipids and tryglicerides, and offer a number of advantages, making them an ideal drug delivery carrier [94]. Adding cationic lipids as surfactants to these dispersed systems makes them suitable for gene delivery. The presence of cationic surfactants, like DOTAP, DOTMA or DC-Chol, causes the formation of positively charged droplets that promote strong electrostatic interactions between emulsion and the anionic nucleic acid phosphate groups [95,96]. For instance, Bruxel et al. [97] prepared a cationic nanoemulsion with DOTAP as a delivery system for oligonucleotides targeting malarial topoisomerase II.

Lipid emulsions are considered to be superior to liposomes mainly in a scaling-up point of view. On the one hand, emulsions can be produced on an industrial scale; on the other

hand, emulsions are stable during storage and are highly biocompatible [94]. In addition, the physical characteristics and serum-resistant properties of the DNA/nanoemulsion complexes suggest that cationic nanoemulsions could be a more efficient carrier system for gene and/or immunogene delivery than liposomes. One of the reasons for the serum-resistant properties of the cationic lipid nanoemulsions may be the stability of the nanoemulsion/DNA complex [98]. However, in spite of extensive research on emulsions, very few reports using cationic amino-based nanoemulsions in gene delivery have been published. After "in vivo" administration, cationic nanoemulsions have shown higher transfection and lower toxicity than liposomes [99].

The incorporation of noninonic surfactant with a branched poly(ethylene glycol), such as Tween 80®, increments the stability of the nanoemulsion and prevent the formation of large nanoemulsion/DNA complexes, probably because of their stearic hindrance and the generation of a hydrophilic surface that may enhance the stability by preventing physical aggregation [94]. In addition, this strategy may prevent from enzymatic degradation in blood, and due to the hydrophilic surface, they are taken up slowly by phagocytic cells, resulting in prolonged circulation in blood [100,101].

3.2.2.3. Solid lipid nanoparticles (SLN)

Solid lipid nanoparticles are particles made from a lipid being solid at room temperature and also at body temperature. They combine advantages of different colloidal systems. Like emulsions or liposomes, they are physiologically compatible and, like polymeric nanoparticles, it is possible to modulate drug release from the lipid matrix. In addition, SLN possess certain advantages. They can be produced without use of organic solvents, using high pressure homogenization (HPH) method that is already successfully implemented in pharmaceutical industry [102]. From the point of view of application, SLN have very good stability [103] and are subject to be lyophilized [104], which facilitates the industrial production.

Cationic SLN, for instance, SLN containing at least one cationic lipid, have been proposed as non-viral vectors for gene delivery [105,20]. It has been shown that cationic SLN can effectively bind nucleic acids, protect them from DNase I degradation and deliver them into living cells. Cationic lipids are used in the preparation of SLN applied in gene therapy not only due to their positive surface charge, but also due to their surfactant activity, necessary to produce an initial emulsion, which is a common step in most preparation techniques. By means of electrostatic interactions, cationic SLN condense nucleic acids on their surface, leading generally to an excess of positive charges in the final complexes. This is beneficial for transfection because condensation facilitates the mobility of nucleic acids, protects them from environmental enzymes and the cationic character of the vectors allows the interaction with negatively charged cell surface. The characteristics of the resulting complexes depend on the ratio between particle and nucleic acid; there must be an equilibrium between the binding forces of the nucleic acids to SLN to achieve protection without hampering the posterior release in the site of action [106]. Release of DNA from the complexes may be one of the most crucial steps determining the optimal ratio for cationic lipid system-mediated transfection [107].

Our research group showed for the first time the expression of a foreign protein with SLNs in an "in vivo" study [108]. After intravenous administration of SLN containing the EGFP plasmid to BALB/c mice, protein expression was detected in the liver and spleen from the third day after administration, and it was maintained for at least 1 week. In a later study [109], we incorporated dextran and protamine in the SLN and the transfection was improved, being detected also in lung. The improvement in the transfection was related to a longer circulation in the bloodstream due to the presence of dextran on the nanoparticle surface. The surface features of this new vector may also induce a lower opsonization and a slower uptake by the RES. Moreover, the high DNA condensation of protamine that contributes to the nuclease resistance may result in an extended stay of plasmid in the organism. The presence of nuclear localization signals in protamine, which improves the nuclear envelope translocation, and its capacity to facilitate transcription [110] may also explain the improvement of the transfection efficacy "in vivo".

SLN have also been applied for the treatment of ocular diseases by gene therapy. After ocular injection of a SLN based vector to rat eyes, the expression of EGFP was detected in various types of cells depending on the administration route: intravitreal or subretinal. In addition, this vector was also able to transfect corneal cells after topical application [111].

SLN may also be used as delivery systems for siRNA or oligonucleotides. Apolipoprotein-free low-density lipoprotein (LDL) mimicking SLN [112] formed stable complexes with siRNA and exhibited comparable gene silencing efficiency to siRNA complexed with the polymer PEI, and lower citotoxicity. Afterwards, Tao et al. [113] showed that lipid nanoparticles caused 90% reduction of luciferase expression for at least 10 days, after a single systemic administration of 3 mg/kg luciferase siRNA to a liver-luciferase mouse model. CTAB stabilized SLN bearing an antisense oligonucleotide against glucosylceramide synthase (asGCS) reduced the viability of the drug resistant NCI/ADR-RES human ovary cancer cells in the presence of the chemotherapeutic doxorubicin [114].

3.2.3. Peptide-based gene non-viral vectors

Many types of peptides, which are generally cationic in nature and able to interact with plasmid DNA through electrostatic interaction, are under intense investigation as a safe alternative for gene therapy [115]. There are mainly four barriers that must be overcome by non-viral vectors to achieve successful gene delivery. The vector must be able to tightly compact and protect DNA, target specific cell-surface receptors, disrupt the endosomal membrane, and deliver the DNA cargo to the nucleus [115]. Peptide-based vectors are advantageous over other non-viral systems because they are able to achieve all of these goals [116]. Cationic peptides rich in basic residues such as lysine and/or arginine are able to efficiently condense DNA into small, compact particles that can be stabilized in serum [117,118]. Attachment of a peptide ligand to a polyplex or lipoplex allows targeting to specific receptors and/or specific cell types. Peptide sequence derived from protein transduction domains are able to selectively lyse the endosomal membrane in its acidic environment leading to cytoplasmic release of the particle [119,120]. Finally, short peptide sequences taken

from longer viral proteins can provide nuclear localization signals that help the transport of the nucleic acids to the nucleus [121,122].

3.2.3.1. Poly-L-lysine

Poly-L-lysine is a biodegradable peptide synthesized by polimerization on N-carboxy-anhydride of lysine [123]. It is able to form nanometer size complexes with polynucleotides owing to the presence of protonable amine groups on the lysine moiety [16]. The most commonly used poly-L-lysine has a polymerization degree of 90 to 450 [124]. This characteristic makes this peptide suitable for "in vivo" use because it is readily biodegradable [116]. However, as the length of the poly-L-lysine increases, so does the cytotoxicity. Moreover, poly-L-lysine exhibits modest transfection when used alone and requires the addition of an edosomolytic agent such as chloroquine or a fusogenic peptide to allow for release into the cytoplasm. An strategy to prevent plasma protein binding and increase circulation half-life is the attachment of poly(ethylene glycol) to the poly-L-lysine [125,126].

3.2.3.2. Peptides in multifunctional delivery systems

Due to the advantages of peptides for gene delivery, they are frequently used to funtionalize cationic lipoplexes or polyplexes. Since these vectors undergo endocytosis, decorating them with endosomolytic peptides for enhanced cytosolic release may be helpful. Moreover, combination with peptides endowed with the ability to target a specific tissue of interest is highly beneficial, since this allows for reduced dose and, therefore, reduced side effects following systemic administration [127]. In a study carried out by our group [19], we improved cell transfection of ARPE-19 cells by using a cell penetration peptide (SAP) with solid lipid nanoparticles. Kwon et al. [128] covalently attached a truncated endosomolytic peptide derived from the carboxy-terminus of the HIV cell entry protein gp41 to a PEI scaffold, obtaining improved gene transfection results compared with unmodified PEI. In other study [20], protamine induced a 6-fold increase in the transfection capacity of SLN in retinal cells due to a shift in the internalization mechanism from caveolae/raft-mediated to clathrin-mediated endocytosis, which promotes the release of the protamine-DNA complexes from the solid lipid nanoparticles; afterwards the transport of the complexes into the nucleus is favoured by the nuclear localization signals of the protamine.

3.3. Physical methods for gene delivery

Gene delivery using physical principles has attracted increasing attention. These methods usually employ a physical force to overcome the membrane barrier of the cells and facilitate intracellular gene transfer. The simplicity is one of the characteristics of these methods. The genetic material is introduced into cells without formulating in any particulate or viral system. In a recent publication, Kamimura et al. [87] revised the different physical methods for gene delivery. These methods include the following:

3.3.1. Needle injection

The DNA is directly injected through a needle-carrying syringe into tissues. Several tissues have been transfected by this method [87]: muscle, skin, liver, cardiac muscle, and solid tumors. DNA vaccination is the major application of this gene delivery system [129]. The efficiency of needle injection of DNA is low; moreover, transfection is limited to the needle surroundings.

3.3.2. Ballistic DNA injection

This method is also called particle bombardment, microprojectile gene transfer or gene gun. DNA-coated gold particles are propelled against cells, forcing intracellular DNA transfer. The accelerating force for DNA-containing particles can be high-voltage electronic discharge, spark discharge or helium pressure discharge. One advantage of this method is that it allows delivering precise DNA doses. However, genes express transiently, and considerable cell damage occurs at the centre of the discharge site. This method has been used in vaccination against the influenza virus [130] and in gene therapy for treatment of ovarian cancer [131].

3.3.3. Electroporation

Gene delivery is achieved by generating pores on a cell membrane through electric pulses. The efficiency is determined by the intensity of the pulses, frequency and duration [132]. Electroporation creates transient permeability of the cell membrane and induces a low level of inflammation at the injection site, facilitating DNA uptake by parenchyma cells and antigen-presenting cells [133]. As drawbacks, the number of cells transfected is low, and surgery is required to reach internal organs. This method has been clinically tested for DNA-based vaccination [134] and for cancer treatment [135].

3.3.4. Sonoporation

Sonoporation utilizes ultrasound to temporally permeabilize the cell membrane to allow cellular uptake of DNA. It is non-invasive and site-specific and could make it possible to destroy tumor cells after systemic delivery, while leave non-targeted organs unaffected [13]. Gene delivery efficiency seems to be dependent on the intensisty of the pulses, frequency and duration [87]. This method has been applied in the brain, cornea, kidney, peritoneal cavity, muscle, and heart, among others. Low-intensity ultrasonund in combination with microbubbles has recently acquired much attention as a safe method of gene delivery [13]. The use of microbubbles as gene vectors is based on the hypothesis that destruction of DNA-loaded microbubbles by a focused ultrasound beam during their microvascular transit through the target area will result in localized transduction upon disruption of the microbubble shell while sparing non-targeted areas. The therapeutic effect of ultrasound-targeted microbubble destruction is relative to the size, stability, and targeting function of microbubbles.

3.3.5. Photoporation

The photoporation method utilizes a single laser pulse as the physical force to generate transient pores on a cell membrane to allow DNA to enter [87]. Efficiency seems to be controlled by the size of the focal point and pulse frequency of the laser. The level of transgene expression reported is similar to that of electroporation. Further studies are needed before this highly sophisticted procedure becomes a practical technique for gene delivery.

3.3.6. Magnetofection

This method employs a magnetic field to promote transfection. DNA is complexed with magnetic nanoparticles made of iron oxide and coated with cationic lipids or polymers through electrostatic interaction. The magnetic particles are then concentrated on the target cells by the influence of an external magnetic field. Similar to the mechanism of non-viral vector-based gene delivery, the cellular uptake of DNA is due to endocytosis and pinocytosis [136]. This method has been successfully applied to a wide range of primary cells, and cells that are difficult to transfect by other non-viral vectors [137].

3.3.7. Hydroporation

Hydroporation, also called hydrodynamic gene delivery method, is the most commonly method used for gene delivery to hepatocytes in rodents. Intrahepatic gene delivery is achieved by a rapid injection of a large volume of DNA solution via the tail vein in rodents, that results in a transient enlargement of fenestrae, generation of a transient membrane defect on the plasma membrane and gene transfer to hepatocytes [87]. This method has been frequently employed in gene therapy research. In order to apply this simple method of gene administration to the clinic, efforts have been made to reduce the injection volume and avoid tissue damage.

4. Strategies to improve transfection mediated by non-viral vectors

The successful delivery of therapeutic genes to the desired target cells and their availability at the intracellular site of action are crucial requirements for efficient gene therapy. The design of safe and efficient non-viral vectors depends mainly on our understanding of the mechanisms involved in the cellular uptake and intracellular disposition of the therapeutic genes as well as their carriers. Moreover, they have to overcome the difficulties after "in vivo" administration.

4.1. Target cell uptake and intracellular trafficking

Nucleic acid must be internalized to interact with the intracellular machinery to execute their effect. The positive surface charge of unshielded complexes facilitates cellular internalization. The non-viral vector can be functionalized with compounds that are recognized by the desire specific target cell type. Peptides, proteins, carbohydrates and small molecules

have been used to induce target cell-specific internalization [138]. For instance, SLN have been combined with peptides that show penetrating properties, such as the dimeric HIV-1 TAT (Trans-Activator of Transcription) peptide [139] or the synthetic SAP (Sweet Arrow Peptide) [19].

Endocytosis has been postulated as the main entry mechanism for non-viral systems. Various endocytosis mechanisms have been described to date: phagocytosis, pinocytosis, clathrin-mediated endocytosis, caveolae/raft-mediated endocytosis and chathrin and caveolae independent endocytosis. Clathrin-mediated endocytosis leads to an intracellular pathway in which endosomes fuse with lysosomes, which degrade their content, whereas caveolae/raft-mediated endocytosis avoids the lysosomal pathway and its consequent vector degradation [20]. Cytosolic delivery from either endosomes or lisosomes has been reported a major limitation in transfection [140]. In consequence, some research groups have used substances that facilitate endosomal escape before lysosomal degradation. For clathrin-mediated endocytosis, the drop in pH is a useful strategy for endosomal scape via proton destabilization conferred by the cationic carrier, or by pH-dependent activation of membrane disruptive helper molecules, such as DOPE or fusogenic peptides [141-143]. More recently, Leung et al. [144] have patented lipids with 4-amino-butiric acid (FAB) as headgroup to form lipid nanoparticles able to introduce nucleic acids, specifically siRNA, into mammalian cells. FAB lipids also demonstrated membrane destabilizing properties.

Once genes are delivered in the cytoplasm they have to diffuse toward the nuclear region. DNA plasmids have difficulties to diffuse in the cytoplasm because they are large in size. Therefore, packaging and complexing them into small particles facilitates its displacement intracellularly. Diffusion is a function of diameter; hence, smaller particles move faster than larger ones. Thus, another way to optimize gene delivery to the nucleus would be to decrease the size of the particles to increase the velocity of passive diffusion through the cytoplasm [145].

The pass through the nuclear membrane is the next step, and it is in general, quite difficult. There are two mechanisms large molecules can use to overcome that barrier: disruption of the nuclear membrane during mitosis, which is conditioned by the division rate of targeted cells, or import through the nuclear pore complex (NPC). This latter mechanism requires nuclear localization signals, which can be used to improve transfection by non-viral vectors [146]. In this regard, protamine is a peptide that condenses DNA and presents sequences of 6 consecutive arginine residues [147], which make this peptide able to translocate molecules such as DNA from the cytoplasm to the nucleus of living cells. Although protamine/DNA polyplexes are not effective gene vectors [148], the combination of protamine with SLN produced good results in both COS-1 and Na 1330 (murine neuroblastoma) culture cells [149,150]. Precondensation of plasmids with this peptide, to form protamine-DNA complexes that are later bound to cationic SLN, is another alternative that has shown higher transfection capacity in retinal cells compared to SLN prepared without protamine [20].

Once inside the nucleus, level of transgene expression depends on the copy number of DNA and its accessibility for the transcription machinery. Studies have shown that the minimum number of plasmids delivered to the nucleus required for measurable transgene expression

depends on the type of vectors [145]. Comparisons between different delivery vehicles showed that higher copy numbers of DNA molecules in the nucleus do not necessarily correlate with higher transfection efficiency. At similar plasmid/nucleus copies, lipofectamine mediated 10-fold higher transfection efficiency than PEI. This suggests that the DNA delivered by PEI is biologically less active than the DNA delivered by lipofectamine. It also emphasizes that a deeper understanding of the nuclear events in gene delivery is required for future progress.

4.2. "In vivo" optimization

Vectors mediating high transfection efficiency "in vitro" often fail to achieve similar results "in vivo". One possible reason is that lipidic and polymeric vectors are optimized "in vitro" using two-dimensional (2D) cultures that lack extracellular "in vivo" barriers and do not realistically reflect "in vivo" conditions. While cells "in vitro" grow in monolayers, cells "in vivo" grow in 3D tissue layers held together by the extracellular matrix [145]. This results in cells with reduced thicknesses but larger widths and lengths. Particles that are taken up directly above the nucleus (supranuclear region) have the shortest transport distance to the nucleus and hence a greater chance of delivery success. The spatiotemporal distribution of carriers, however, determines the optimal time for endosomal escape and the optimal intracellular pathway [151]. This highlights the need to develop adequate "in vitro" models that mimics as much as possible the "in vivo" conditions to optimize carriers for gene therapy.

After intravenous administration, plasma nuclease degradation of the nucleic acid is the first barrier that needs to be overcome for therapeutic nucleic acid action. Nucleic acids can be degraded by hydrolytic endo- and exo-nucleases. Both types of nucleases are present in blood. Therefore, increasing nuclease resistance is crucial for achieving therapeutic effects. Naked nucleic acids are not only rapidly degraded upon intravenous injection, they are also cleared from the circulation rapidly, further limiting target tissue localization [138]. To improve nuclease resistance and colloidal stability, complexation strength is an important factor. Shielding the non-viral vectors with poly-L-lysine or poly(ethylene glycol), as mentioned previously, prolongs the circulation time in blood of the vectors.

Vectors delivered "in vivo" by systemic administration not only have to withstand the bloodstream, but also have to overcome the cellular matrix to reach all cell layers of the tissue. While large particles seem to have an advantage "in vitro" due to a sedimentation effect on cells, efficient delivery of particles deep into organs requires particles <100 nm. Small particles (40 nm) diffuse faster and more effectively in the extracellular matrix and inner layers of tissues, whereas larger particles (>100 nm) are restricted by steric hindrance [152].

The net cationic charge of the synthetic vector is a determinant of circulation time, tissue distribution and cellular uptake of synthetic vectors by inducing interactions with negatively blood constituents, such as erythrocytes and proteins. The opsonisation of foreign particles by plasma proteins actually represents one of the steps in the natural process of removal of foreign particles by the innate immune system [153]. This may result in obstruction of small capillaries, possibly leading to serious complication, such as pulmonary embolism [154]. Part of the complexes end up in the reticuloendothelial system (RES), where they are re-

moved rapidly by phagocytosis or by trapping in fine capillary beds [155]. The nanocarriers, when circulating in blood, can activate the complement system and it seems that the complement activation is higher as the surface charge increases [156,157].

The interaction with blood components is related to the intrinsic properties of the cationic compound (side chain end groups, its spatial conformation and molecular weight), as well as the applied Nitrogen:Phosphate (N:P) ratio [138]. Shielding of the positive surface charge of complexes is currently an important strategy to circumvent the aforementioned problems. The most popular strategy is based on the attachment of water-soluble, neutral, flexible polymers, as poly(ethylene glycol), poly(vinylpyrrolidone) and poly(hydroxyethyl-L-asparagine). The efficacy of the shielding effect of these polymers is determined by the molecular weight and grafting density of the shielding polymer [158]. Longer chains are usually more effective in protecting the particle (surface) from aggregation and opsonisation.

The nanocarriers must arrive to the target tissue to exert their action. Although most commonly used targeting strategies consist of proteins and peptides, carbohydrates have also been utilized [159]. The access of non-viral vector to tumors has been investigated extensively. The discontinuous endothelial cell layer has gaps that give the nanocarriers the opportunity to escape the vascular bed and migrate into the tumoral mass. The most common entities used for tumor targeting include transferrin, epidermal growth factor, and the integrin-binding tripeptide arginine-glycine-aspartic acid (RGD) [159]. Brain targeting has also a great interest; most gene vector do not cross the blood-brain barrier (BBB) after intravenous administration and must be administered through intracerebral injection, which is highly invasive and does not allow for delivery of the gene to other areas of the brain. Injection in the cerebrospinal fluid is also another strategy. Commonly used ligands for mediated uptake are insuline-like growth factors, transferrin or low-density lipid protein [159]. Targeting to the liver has been also investigated in a great extension by many researchers. Carbohydrate-related molecules, such as galactose, asialofetuin, N-acetylgalactosamine and folic acid are the most commonly molecules used for liver targeting [159]. Targeting to endothelial cells provides avenues for improvement of specificity and effectiveness of treatment of many diseases, such as cardiovascular or metabolic diseases [160]. Among other endothelial cell surface determinants, intercellular adhesion molecule-1 (CD54 or ICA-1, a 110-KDa Ig-like transmembrane constitutive endothelial adhesion molecule) is a good candidate target for this goal. ICAM-1 targeting can be achieved by coupling Anti-ICAM-1 antibodies to carriers [161].

5. Conclusion

The success of gene therapy is highly dependent on the delivery vector. Viral vectors have dominated the clinical trials in gene therapy for its relatively high delivery efficiency. However, the improvement of efficacy of non-viral vectors has lead to an increased number of products entering into clinical trials. A better understanding of the mechanisms governing the efficiency of transfection, from the formation of the complexes to their intracellular delivery, will lead to the design of better adapted non-viral vectors for gene therapy applica-

tions. A number of potentially rate-limiting steps in the processes of non-viral-mediated gene delivery have been identified, which include the efficiency of cell surface association, internalization, release of gene from intracellular compartments such as endosomes, transfer via the cytosol and translocation into the nucleus and transcription efficacy. Insight into molecular features of each of these steps is essential in order to determine their effectiveness as a barrier and to identify means of overcoming these hurdles. Although non-viral vectors may work reasonably well "in vitro", clinical success is still far from ideal. Considering the number of research groups that focus their investigations on the development of new vectors for gene therapy, together with the advances in the development of new technologies to better understand their "in vitro" and "in vivo" behavior, the present limitations of non-viral vectors will be resolved rationally.

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